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DOI:
10.1007/s10456-014-9448-z

Document Version
Peer reviewed version

Citation for published version (Harvard):
Zhuang, X, Ahmed, F, Zhang, Y, Ferguson, H, Steele, JC, Steven, NM, Nagy, Z, Heath, VL, Toellner, K-M & Bicknell, R 2015, 'Robo4 vaccines induce antibodies that retard tumor growth', *Angiogenesis*, vol. 18, no. 1, pp. 83-95. https://doi.org/10.1007/s10456-014-9448-z

Robo4 vaccines induce antibodies that retard tumor growth

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Running title: Vaccination against tumor vessels

Word count: 4629

Abstract

Tumor endothelial specific expression of Robo4 in adults identifies this plasma membrane protein as an anti-cancer target for immunotherapeutic approaches, such as vaccination. In this report, we describe how vaccination against Robo4 inhibits angiogenesis and tumor growth. To break tolerance to the auto-antigen Robo4, mice were immunised with the extracellular domain of mouse Robo4, fused to the Fc domain of human immunoglobulin within an adjuvant. Vaccinated mice show a strong antibody response to Robo4, with no objectively detectable adverse effects on health. Robo4 vaccinated mice showed impaired fibrovascular invasion and angiogenesis in a rodent sponge implantation assay, as well as a reduced growth of implanted syngeneic Lewis lung carcinoma. The anti-tumor effect of Robo4 vaccination was present in CD8 deficient mice but absent in B cell or IgG1 knockout mice, suggesting antibody dependent cell mediated cytotoxicity as the anti-vascular/anti-tumor mechanism. Finally, we show that an adjuvant free soluble Robo4-carrier conjugate can retard tumor growth in carrier primed mice. These results point to appropriate Robo4 conjugates as potential anti-angiogenic vaccines for cancer patients.

Word count: 172

Key words: immunotherapy, conjugate vaccines, ADCC, cancer

Introduction

The tumor microenvironment is markedly different from that of a healthy tissue in being hypoxic, acidic and because of poor vessel development, showing reduced blood perfusion and shear stress. We have previously shown that low shear stress at the endothelial surface in tumor vessels induces expression of specific proteins including Robo4 and CLEC14A (1). Proteins induced in endothelium by the tumor microenvironment have been designated tumor endothelial markers (TEM's). Studies involving TEM's have shown that their targeting can impede tumor growth in animal models (2). TEM's localised at either the plasma membrane or secreted into the extracellular matrix are accessible for antibody targeting, which can be either infused or generated *de novo* via vaccination. An advantage of active vaccination is the possibility of generating both antibody production *in situ* and cytotoxic T-cell mediated immunity. Recent work in experimental mouse models has shown reduction in tumor growth after vaccination with endothelial expressed proteins, including the vascular endothelial growth factor receptor (VEGFR) -2, Endoglin/CD105, Delta-like 4 (DLL4) and the extra domain-B of fibronectin (reviewed in (2), (3-5))

For effective immunotherapy, the choice of tumor endothelial marker is critical. Robo4 has recently been shown to be a superior target to VEGFR2 (6). In that study, Mai *et al.* demonstrated that while internalising antibodies targeting VEGFR2 or Robo4 both retard tumor growth, there was significant toxicity associated with those targeting VEGFR2 but not with those targeting Robo4 (6). This is likely to reflect the expression of VEGFR2 on endothelium in healthy tissue compared with Robo4 that is restricted to the tumor endothelium (1, 7-10). An endothelial specific member of the Roundabout family of guidance molecules, Robo4 is present on the vessels of a number of tumor types including pancreatic,

bladder, lung and prostate cancer (1, 7-10). Roles for Robo4 have been identified in angiogenic sprouting and filopodia formation as well as maintenance of the endothelial barrier through binding of its ligand Unc5b (reviewed in (11)).

Robo4 as an autoantigen does not easily raise T cell help. We therefore linked Robo4 to foreign proteins. This efficiently induced autoantibodies specific to Robo4. We show that a carrier priming followed by carrier-conjugate vaccination protocol efficiently breaks B cell tolerance to Robo4 and induces an anti-tumor vessel immune response based on Robo4-specific IgG1. Vaccination with the extracellular domain of Robo4 affects vascularisation of sponges implanted into vaccinated mice and retards the growth of subcutaneously implanted tumors. This anti-tumor effect in the absence of adverse effects makes targeting Robo4 a promising strategy for the treatment of human cancer.

Materials and methods

Blood sampling

Fresh blood from healthy donors or cancer patients was collected with ethical approval (South Birmingham Ethics Committee REC ref 06/Q2707/338) and patient consent.

Mice

Mice were on a C57/BL6 background and were either wild type, B cell deficient strain with a targeted deletion of Jh (12) or IgG1 deficient strain (13). All animal experimentation was carried out in accordance with British Home Office License number PPL 80/2217 held by RB.

Cell culture

293T and Lewis lung carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % foetal calf serum (FCS) and 4 mM L-glutamine. Cells were passaged at confluence.

Production of lentivector and expression of mouse Robo4-Fc using a lentiviral system

Mouse Robo4-Fc was amplified from a parent vector by PCR using Phusion proof-reading polymerase (New England Biolabs, MA, USA) with the following primers:

Forward: 5’-TAGTAGATTTAAATACCATGGGCTCTGGAGGAACG-3’

Reverse: 5’-TAGTAGTTAATTAATCATTTACCCGGAGACAGGGAGAG-3’

The amino acid sequence of mouse Robo4-Fc is shown in **Supplemental figure 1**. Second generation lentivirus stocks were produced by calcium phosphate transfection into HEK293T cells with the packaging (psPAX2, Didier Trono Addgene plasmid 12260) and lentivector (pWPI vector, Didier Trono, Addgene plasmid 12254) together with a plasmid (PMD2.G, Didier Trono Addgene plasmid 12259) which encodes the VSVG envelope protein (14, 15). Briefly, for one 10 cm plate of 293T cells, 3 x 10^6 cells were seeded 24 hours prior to transfection. 20 μg lentivector, 15 μg packaging plasmid (2nd generation, psPAX2) and 6 μg envelope plasmid (PMD2.G) were used. The transfection mix was prepared by adding the plasmids to 450 μl distilled water and 63 μl of 2M calcium chloride. 500 μl 2x HEPES-buffered saline (HBS, pH 7.05) was then added. After 15 min at room temperature the entire mixture was added to the plate. The medium was replaced after 6 – 8 hours with fresh DMEM/10% FCS. The viral supernatant was collected 48 hours later, filtered through a 0.45 μm filter. Virus was used for transduction or stored at -80^{o}C for future use. To transduce the 293T cells, 3 ml of the viral supernatant was added to 3 x 10^6 cells at 37^{o}C for 7 hours and then replaced with DMEM/10%FCS. Since the lentivector pWPI contained an IRES linked GFP sequence, the transduced cells were FACs sorted for GFP to obtain a pure population of Robo4 producing cells.

Affinity purification of Fc fusion proteins using a Protein A column

The FACS sorted lentivirus transduced 293T cells (mRobo4-Fc-pWPI) were expanded with complete media in 15 cm tissue culture dishes. When the confluency reached 80%, media was replaced with fresh OptiMEM (Invitrogen, Paisley, UK). 5 collections within 10 days gave 2~3 L of conditioned media. Collected media was adjusted to pH 8.0. 1 mM EDTA and a few phenylmethylsulfonyl fluoride crystals were added to the conditioned media to inhibit

proteases. Conditioned media was then run through a Hi-Trap protein A column at 1 ml/min at 4^{o}C followed by a 5x column volume wash with pH 7 buffer (Na_2PO_4, 20 mM). A pH gradient elution was achieved by FPLC chromatography using pH 3 buffer (sodium citrate 100 mM). The major products were eluted between pH 3.5 and 4, the pH adjusted back to 7 using Tris-HCl pH 9.5 solution and stored at 4^{o}C.

Papain cleavage of recombinant human and mouse Robo4-Fc and depletion of the Fc fragment.

Robo4-Fc was cleaved by incubation with the cysteine protease papain (Sigma, Gillingham, UK) as follows: 600 µg/ml Robo4Fc, 1.6 µg/ml papain in 0.15 M NaCl, 0.67 mM EDTA and 0.5 mM L-cysteine. Cleavage was performed at 37^{o}C for 40 minutes. 50 µl of iodoacetic acid (130 mM, pH 6.8) was added to quench the reaction (16, 17). Based on an early study investigating papain cleavage site of human IgG (18), it is most likely that papain cuts the histidine-threonine (HT) bond within the Robo4-Fc fusion protein (**Supplemental figure 1**). To remove the Fc fragments, 50 µl of PBS washed protein G beads (Sigma, Gillingham, UK) was added to 1 ml of reaction mix and placed on a wheel and rotated at 4^{o}C for 2 hours. Protein G beads were removed by centrifugation, the supernatant collected and analysed by Western blot against polyclonal anti Robo4 (Abcam, Cambridge, UK) and anti human Fc antibody (Sigma, Gillingham, UK) to confirm successful cleavage and Fc depletion. Supernatants containing pure Robo4 protein were stored at 4^{o}C.

Measurement of Robo4 antibodies in human and mouse serum

An ELISA plate was coated with papain cleaved human or mouse Robo4 overnight at 4^{o}C. 5 µl of serum from cancer patients, healthy individuals or immunised mice was diluted ten-fold in PBS and applied to an ELISA plate. Peroxidase conjugated anti-human IgG or anti-mouse IgG antibody (Sigma, Gillingham, UK) was applied to each well as the secondary antibody and the rest of the procedure was as described above. To determine the antibody subclasses in serum from vaccinated mice, ELISA plates coated with pure mouse Robo4 protein were incubated with ten-fold diluted mouse serum following addition of AP conjugated anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM secondary antibodies (Southern Biotech, Cambridge, UK).

Immunohistochemistry and immunofluorescence of paraffin embedded tissue

Immunohistochemistry and immunofluorescence was performed as previously described (1). Briefly, the Lewis lung carcinoma paraffin embedded tissue sections were incubated with 1/100 CD31 (JC70) monoclonal antibody (Dako, Glostrup, Denmark) and 1/500 Robo4 polyclonal antibody (Abcam, Cambridge, UK) for 1 hour. Following a TBS-0.1% Tween-20 wash, the sections were visualised using Vector Labs Vector ImmPRESS universal secondary antibody kit (30 min) and Vector ImmPACT DAB chromogen (10 min). Finally sections were counterstained in Meyer's haematoxylin, dehydrated, cleared and mounted in distyrene-plasticizer-xylene (DPX, Surgipath, Peterborough, UK). To investigate co-localization of Robo4 and *Bandieria simplicifolia* isolectin B4 in resected sponges, slides were incubated in 10 µg/ml of Robo4 polyclonal antisera (Abcam, Cambridge, UK). Mouse endothelium was visualized with 5 µg/ml of isolectin B4 conjugated to FITC (Sigma, Gillingham, UK). For fibrinogen and neutrophil staining, Lewis lung carcinoma sections were treated with 1 in 500 diluted polyclonal fibrinogen antisera (Dako, Glostrup, Denmark) or 1 in 1000 diluted

monoclonal anti-Ly6G and Ly-6C antibody (BD PharmingenTM, Oxford, UK). After overnight incubation at 4^{o}C, sections were probed with 15 µg/ml of rhodamine or FITC conjugated secondary antibody (Invitrogen, Paisley, UK). Slides were permanently mounted with Prolong gold anti-fade reagent including DAPI (Invitrogen, Paisley, UK) to counterstain cell nuclei. Sections were then examined using an Axiovert 100M laser scanning confocal microscope (Carl Zeiss, Welwyn Garden City, UK).

Mouse immunisation with Freund's and alum adjuvants

Mice were immunised subcutaneously with 50 µg of Robo4 protein in Freund's complete adjuvant followed two weeks later with 50 µg in incomplete Freund's adjuvant. For alum adjuvant, the same volume of Robo4 and 9% aluminium potassium sulphate were mixed. The pH was adjusted to 6.5, incubated in the dark for 1hr at room temperature, and then the mix was washed twice with PBS. The antigen-alum precipitate was finally resuspended in an appropriate volume and 50 µg of antigen was injected intraperitoneally per mouse. 20 µl of blood was collected from each mouse before injection. The level of Robo4 specific antibody was tested by ELISA assay using papain cleaved mouse Robo4 as the coating protein. Organs including brain, heart, lung, liver, kidney and spleen from both groups were collected and fixed in 4% formalin. The tissues were then paraffin embedded, sectioned and stained with haematoxylin and eosin. Pictures from the stained sections were acquired using a Leica DM IL microscope (Leica, Milton Keynes, UK) and a USB 2.0 2M Xli camera (XL Imaging LLC, Carrollton, TX, USA).

Depletion of the CD8^{+} T cell subset in mice

Depletion of $CD8^+$ T cells from Fc or Robo4-Fc vaccinated mice was initiated 14 days post-immunisation. In brief, 200 µg of rat anti-mouse CD8 (an equal concentration of YTS 169 anti-mouse CD8 alpha and YTS 156.7.7 anti-mouse CD8 beta, kindly supplied by Professor Steve Cobbold, Therapeutic Immunology Group, Sir William Dunn School of Pathology, University of Oxford, UK) was injected intraperitoneally per mouse every third days for the duration of the experiment (19, 20). Depletion of the CD8 T cell subset was verified by flow cytometry analysis of the peripheral blood cells for CD3 and CD8 (BD Biosciences, Stockholm, Sweden).

Immunisation with Robo4 conjugates in Fc or CGG primed mice

For antigen priming, 50 µg of human Fc protein or chicken γ globulin (CGG, Sigma, Gillingham, UK) per mouse was delivered intraperitoneally with alum adjuvant. Purified mouse Robo4-Fc protein was cross-linked to CGG using glutaraldehyde as previously described (21). 50 µg of Robo4-Fc or Robo4-Fc-CGG conjugate was intraperitoneally injected into 5-week primed mice. Pure Fc protein or Fc-CGG conjugate were used as controls.

Sponge implantation and wound healing assays

The mouse subcutaneous sponge angiogenesis assay was performed as previously described (22). Briefly, each C57/BL6 mouse received a sponge and 200 µl of 10 ng/ml of bFGF was injected into the sponge on alternate days. On day 14, sponges were excised, fixed in 4% formalin followed by 70% ethanol. Fixed sponges were then paraffin embedded, sectioned and haematoxylin and eosin stained. The area of invasion compared to that of the whole

sponge was obtained using ImageJ software and the percentage of invaded area calculated. To quantify wound healing, images were taken using a Leica microscope (DM6000) and the open wound areas were quantified using ImageJ software at each time point.

Tumor growth experiments

C57/BL6 mice were subcutaneously implanted with 1 x 10^6 of Lewis Lung Carcinoma cells. Tumor size was measured at indicated days and tumor volume calculated following the formula: length x $width^2$ x 0.4 (23). ANOVA analysis was performed for the comparison of tumor growth between Robo4 and Fc vaccinated mice.

Results

Vaccination with Robo4 protein generates an antibody response in mice

To measure Robo4 specific antibodies in sera from mouse and man, an enzyme linked immunoabsorbant assay (ELISA) was developed using the extracellular domain of mouse or human Robo4 immobilized on a microtitre plate. Robo4 was produced as the extracellular domain of mouse or human Robo4 fused to the Fc domain of human immunoglobulin (Robo4-Fc) (**Supplemental figure 2a**). The Fc domain was cleaved from the fusion protein by papain, and the Fc portion removed using protein A beads (**Supplemental figure 2b**). The detection limit of the assay was found to be an antibody concentration of 8 ng/ml (**Supplemental figure 2c**). To eliminate the possibility of a preexisting Robo4 antibody in cancer patients due to its high expression in tumour vessels, we looked for the presence of Robo4 specific antibodies in sera of both cancer patients (n = 20) and non-cancer individuals (n = 100). Our investigation confirmed that no immunoreactivity was detected from either patient sera or from healthy control sera (data not shown). The lack of an antibody response to Robo4 in cancer patients may be due to inadequate antigen presentation, immune suppression, or the lack of specific T cell help due to central tolerance. The absence of antibodies to Robo4 in cancer patients justified attempts to vaccinate against Robo4 and to document the effect on angiogenesis and tumor growth.

With some self-antigens, it is possible to break tolerance by conjugating to a foreign antigen and immunising in combination with a potent adjuvant. Immunisation of mice with 50 μg of a mouse Robo4 human Fc fusion protein (Robo4-Fc) in complete Freund's adjuvant followed by boosting with Robo4 in incomplete Freund's adjuvant (**Figure 1a**) resulted in a robust antibody response (**Figure 1b**). Immunisation without adjuvant alone gave no antibody

response (data not shown). Characterising the isotypes of the induced antibodies revealed that there were high levels of IgG1, with lower titres of IgG2a, IgG2b and IgG3 (**Figure 1c**). Immunisation with protein antigens in mice tends to induce immunoglobulin class switching predominantly to IgG1 associated with T-helper 2-type immunity, even in the presence of Th1 polarizing bacterial adjuvants (24, 25).

Robo4 vaccination inhibits vascularisation of a subcutaneously implanted sponge

To determine whether the induced Robo4 antibodies would affect angiogenesis, sponges were subcutaneously implanted into vaccinated mice. The implanted sponges were injected with pro-angiogenic basic fibroblast growth factor (bFGF) on alternate days, and harvested after fourteen days. In Fc vaccinated control mice, Robo4 expression was observed on the newly formed vessels, and co-localised with the endothelial-specific glycosylation selectively recognised by *Bandieria simplicifolia* isolectin B4 - FITC (26) (**Supplemental figure 3a**). Robo4 vaccination dramatically reduced the vascularisation compared to control Fc immunised mice. Quantitation of fibrotic invasion showed a highly significant reduction from a mean of 78% in control mice to 36% in Robo4 vaccinated mice (**Figure 2a and 2b**). Evaluation of vessel density and dimensions showed that Robo4 vaccination significantly reduced both these parameters compared to control mice (**Figure 2c-e**). This data shows that an immune response to Robo4 impeded the development of new blood vessels within the sponge.

Robo4 protein vaccination reduces tumor growth and disrupts tumor vessels

Since angiogenesis promotes growth of solid tumors, the effect of Robo4 vaccination on the growth of subcutaneously implanted mouse tumor cells was examined. Lewis lung carcinoma cells form tumors with Robo4 expressing vessels (**Supplemental figure 3b**). Tumor growth was significantly retarded in Robo4 immunised mice compared with control Fc immunised mice (**Figure 3a**). In order to elucidate the effect of the immune response to Robo4 on the tumor vessels, immunofluorescent staining of fibrinogen was performed on tumor sections since deposition of this protein is an indicator of increased vascular leakage (27). There was significantly increased staining of fibrinogen in tumors from the Robo4 vaccinated group, consistent with vessel damage (**Figure 3b**). Immunostaining with antibodies to a neutrophil-specific marker showed an increased neutrophil infiltration into the Robo4 vaccinated group compared with a control Fc immunised group (**Figure 3c**). This confirmed increased tumor inflammation in the tumors in the Robo4-Fc vaccinated mice.

Robo4 vaccination with alum adjuvant reduces tumor growth via an antibody-mediated mechanism

While Freund's complete adjuvant is a good inducer of cytotoxic T cell responses, it has severe side reactions and is therefore not permissible for use in humans (25, 28). To identify a milder alternative we tested alum based adjuvants, which are commonly used in human vaccination, but mainly primes for Th2-dependent antibody responses. We initially compared Robo4 antibody titers in the serum of alum and Freund's vaccinated mice. With a single dose of alum vaccination, a similar level of Robo4 antibody was achieved compared to that induced by Freund's adjuvant (**Supplemental figure 4**). Mice were immunised with Fc or Robo4-Fc protein alum precipitate intraperitoneally and Lewis lung carcinoma cells were

injected subcutaneously on the same day. Growth of tumor was again retarded in Robo4 vaccinated mice to a similar extent to pre-vaccination with Freund's adjuvant (**Figure 4a**).

To investigate the mechanism of the therapeutic effect elicited by Robo4 in alum vaccination, we examined the involvement of B- or cytotoxic T-lymphocytes. To determine the role of cytotoxic T cells in the anti-tumor effect, $CD8^+$ T cells were depleted by injection of antibodies 14 days after Robo4-Fc or Fc immunisation. Efficient depletion was verified by flow cytometry of peripheral blood cells from the vaccinated mice (**Supplemental figure 5**). This showed that in the absence of $CD8^+$ T cells the therapeutic effect of Robo4 vaccination was unchanged, arguing against a role for cytotoxic T cells in the inhibition of tumor growth (**Figure 4c**). Robo4 specific antibodies were confirmed 21 days post-immunisation (**Figure 4b and d**). In contrast, similar experiments performed in genetically modified B cell deficient mice lacking the whole antibody repertoire (12), or B cell sufficient mice that only lack the IgG1 subclass (13) completely abolished the inhibitory antitumor effect of vaccination (**Figure 4e and f**). Robo4 antibody was undetectable in serum from either B cell or IgG1 deficient mice (data not shown). These results suggest that the humoral response to Robo4 and in particular IgG1 has a key role in the inhibition of tumor growth.

Soluble Robo4 conjugated to a carrier protein induces a rapid protective antibody response in the absence of an adjuvant

Prior vaccination with a carrier protein in an adjuvant ensured a high frequency of carrier-specific memory T-cells to provide help to autoantigen-specific B cells. This strategy may lead to efficient and rapid onset of antigen-specific antibody production, which develops

faster than primary responses in naïve animals (**Figure 5a**) (29, 30). Here, we explored the feasibility of using this strategy to induce rapid immune responses to Robo4. Firstly, intraperitoneal immunisation with alum precipitated human Fc was used to induce a human Fc specific immunological memory. Five weeks later, mice were challenged by intraperitoneal immunisation with 50 μg of soluble mouse Robo4 fused to human Fc, or pure soluble human Fc protein. Tumor cells were concurrently implanted. Fc priming resulted in a strong tumor growth inhibition in the Robo4-Fc vaccinated animals (**Figure 5b**). To further explore this approach for cross-linked antigens, mice were primed with alum precipitated chicken gamma globulin (CGG) five weeks prior to immunisation with soluble Robo4-Fc which was chemically cross-linked to CGG. Similarly to the Fc-primed mice, vaccination of CGG-primed animals with cross-linked Robo4-Fc-CGG led to significant and sustained tumor growth inhibition (**Figure 5d**). Anti-Robo4 antibody titers in these two experiments were verified in serum samples from vaccinated mice 21 days post-vaccination (**Figure 5c and e**).

Vaccination against Robo4 has no affect on wound healing or organ integrity

The potential for impaired wound healing or organ-specific pathology following Robo4 vaccination was investigated. Freund's adjuvant was used in this experiment since it is reported to induce the strongest antibody responses. Quantitation of surgically created wounds on alternate days showed no difference between the Robo4 vaccinated and control Fc vaccinated groups. Wounds were completely healed on day 7 in all animals (**Figure 6a**). Haematoxylin and eosin staining of tissue collected from Robo4-Fc and Fc control vaccinated mice 6 weeks post-vaccination showed no observable pathology (**Figure 6b**).

Discussion

The tumor vasculature is a major target for anti-cancer therapies that range from anti-angiogenesis to inflicting damage on established tumor vessels. Disruption of tumor vessels has been achieved with both toxin conjugated antibodies and low molecular weight species such as combretastatin (31). Antibody directed approaches have shown particular promise and a natural progression to the use of ectopically delivered antibodies is to generate an immune response to a target *in vivo.* Indeed there have been several attempts to vaccinate against tumor vessels using targets such as delta-like 4 (3) and the EDB domain of fibronectin ((4), reviewed in (2)). We have recently shown that the endothelial specific protein Robo4 is induced by low shear stress on the endothelial surface (1). Induction by low shear stress leads to Robo4 expression on poorly differentiated and perfused tumor vessels but absence in the well perfused vessels of healthy tissue (11). In this study we have explored the possibility of harnessing Robo4 as a tumor vessel specific antigen.

Robo4 is an endothelial specific member of the roundabout gene family (8, 32, 33). The function of Robo4 is unclear but appears to depend on the context of the endothelial cell. In tip cells, Robo4 promotes filopodia formation and cell migration and is generally pro-angiogenic (34). In contrast, in the resting 'palisade' cell Robo4 stabilises the endothelial barrier (35), possibly by ligation of UNC5B on an adjacent endothelial cell (36). While weak Robo4 expression is found on all endothelium (37), expression is much stronger on tumor endothelium (1, 8, 9). For example, high expression of Robo4 is found in over 60% of pancreatic, prostate, stomach, lung and renal cancers pointing to a promising target in cancer.

A key consideration for vascular targeting strategies is the effect these treatments might have on the vasculature in healthy tissue. While in the HUVEC-immunised patients no adverse effects were observed, gastrointestinal bleeding was observed in a pancreatic cancer trial using vaccination to peptides from VEGFR-1 (38). Given the importance of VEGFR in tumor angiogenesis, and the emergence of VEGF-targeted therapies such as bevacizumab, significant effort has gone into immune targeting of this signalling pathway (reviewed in (2)). Indeed prolonged use of VEGF-based anti-angiogenics has highlighted a role for VEGF signalling in the maintenance of the normal vasculature (39). Though bevacizumab has been clinically effective, in some cases wound healing has been adversely affected (40). This does not appear to be the case in Robo4 vaccinated mice where there was no delay in wound healing after sponge implantation. Histological analysis of heart, lung, kidney, spleen, brain or liver showed no differences between control and Robo4 vaccinated mice, which all suggest that targeting Robo4 may be a safe immunotherapeutic intervention. Induced expression of Robo4 in tumor vessels has been exploited to enable tumor vessel imaging (41) and as a target for antibody mediated anti-tumor therapy (6). The latter study showed that internalizing antibodies to either VEGFR2 or Robo4 induced a strong anti-tumor effect in mouse cancer models. A significant finding of the study was that while antibodies to VEGFR2 caused extensive toxicity, those to Robo4 were without adverse effects. These results are consistent with the lack of pathology seen with tumor blocking anti-Robo4 antibodies described by others and contrast to the lethal toxicity of anti-VEGFR2 antibodies (6).

It was not possible in our hands to culture Robo4 specific T cells from human PBMCs (unpublished data), nor could we detect Robo4-specific antibodies in healthy people or cancer patients. In view of this lack of immune activation, it was considered worthwhile to attempt Robo4 vaccination. As seen for other antigens (25), it was possible to efficiently

vaccinate using Robo4 with Freund's complete adjuvant. Vaccinated mice showed a strong anti-angiogenic response and retarded growth of Lewis lung tumors. Tumors in the Robo4 vaccinated mice showed increased vascular damage and inflammation. As Freund's adjuvant has severe side effects, alum adjuvant was also tested.~~,~~ The literature generally reports that alum is a poor adjuvant compared to Freund's complete adjuvant for breaking tolerance (42). Thus, the equally strong response to Robo4 with both adjuvants was somewhat unexpected. ~~despite of which is generally considered a weaker adjuvant to self-antigens compared with Freund's (42). A comparable level of Robo4 antibody titer was seen in the sera from alum and Freund's adjuvant vaccinated mice.~~ This ~~could be possibly explained by~~may be related to the fact that Robo4 is an embryonic protein that is absent ~~from~~ in healthy adults and only ~~present~~ expressed in tumour vessels. Although alum vaccination preferentially induces Th2 type CD4 T cell activation and IgG1 responses (30), similar tumor growth inhibition was observed. A potential mechanism for the anti-angiogenic/anti-tumor effect was then explored. Equivalent Robo4 vaccine induced tumor growth inhibition was seen in $CD8^+$ depleted mice but lost in the absence of B cells, suggesting that the anti-tumor effect of vaccination is mainly antibody and not cytotoxic T cell mediated. While others showed a major role of cyototoxic T cells and not antibody in the anti-tumor effect of OVA in Freund's adjuvant vaccination (28), this effect was measured on OVA expressing transfectoma cells, where it is unlikely that native antigen is expressed on the cell surface. Observations similar to ours have been reported for a DNA vaccine against the endothelial tip cell marker Delta-like 4, which showed that upon depletion of $CD8^+$ T cells, the anti-tumor effect was not impaired in DLL4 vaccinated mice (3).

Another reason for the use of strong adjuvants is the need to overcome T cell tolerance to self-antigens (43). We circumvented this problem by using a conjugate vaccine approach, where mice pre-vaccinated with carrier protein (human Fc or CGG) were challenged with the

corresponding Robo4 conjugate in free form. This approach efficiently induces an anti-tumor effect without further adjuvant and should be transferable to combinations of human Robo4 with carriers used in human vaccines given during childhood vaccination such as diphtheria toxoid (44). Conjugate vaccines have been used against tumor neoantigens, although not in the presence of preexisting T-cell memory (45). How vaccination with a combination of autoantigen and foreign carrier impacts on the longevity of the antibody response, e.g. through recruitment of regulatory T cells, requires further investigation. It has been found by others that vaccination to autoantigens tends to induce considerably shorter lasting immune responses (25), which may improve long term safety of this approach.

Robo4-specific antibodies are likely to inhibit tumor growth through antibody dependent cell mediated cytotoxicity (ADCC) or complement dependent lysis. Surprisingly, IgG1 deficiency results in a major reduction in the tumor inhibition following Robo4 vaccination. It is likely that Robo4 vaccination of IgG1 deficient mice results in compensatory production of other antibody classes through switching to other IgG subclasses and IgM from non-switched B cells. Thus complement dependent lysis is an unlikely candidate as the mechanism for the vaccine induced tumor suppression. It is of interest to consider why IgG1 could be a key mediator of the anti-tumor effect. It has been shown that IgG1 binding to FcγRIII mediates tumor-specific ADCC in phagocytic cells (46). In fact many of today's tumor-specific antibody drugs are derived from murine IgG1 antibodies. IgG1 has also been shown to play a major role in protection from some pathogens (47). However, IgG2a antibodies can activate tumor-specific ADCC in a similar way (46), and no Fc receptors with affinity specifically for IgG1 are known that would mediate this effect (48). Therefore, the potency of IgG1 in

immune protection may be due to an undefined IgG1 specific Fc receptor, or the specificity and affinity of germinal centre derived IgG1 is a key mediator of the protective effect.

A caveat for this study is that human and mouse IgG subclasses are not evolutionary homologues and may not function in the same way. It has been shown that Th2 induced human IgG4, which together with human IgG1 is the closest homologue to murine IgG1, impairs vaccine induced tumor protection in man (49). We conclude that it remains to be tested whether this approach will work in a similar fashion in the clinic.

Acknowledgements

This work was funded by Cancer Research UK grant number C4719/A7825 awarded to Roy Bicknell.

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Figure legends

Figure 1

Vaccination of mice with mouse Robo4 generated a Robo4-specific IgG response

(a) Protocol for Robo4 vaccination prior to sponge or tumor implantation. 50 µg of Robo4-Fc or control Fc protein was subcutaneously injected into mice in complete Freund's adjuvant (CFA) on day 0. Mice were then boosted with the same amount of protein in incomplete Freund's adjuvant (IFA) at day 14. Vaccinated mice either received sponge implantation or tumor cell inoculation on day 28. **(b)** Vaccination with Robo4-Fc induces Robo4-specific antibody production. Mice (C57/BL6) were vaccinated with Fc or mouse Robo4-Fc protein injection according to the protocol in (a). Robo4 antibodies were determined by ELISA at day 14 and day 28. The mean and standard error of the mean (SEM) are indicated, (n = 6 per group, $p < 0.001$, Mann-Whitney test). **(c)** Determination of the antibody isotypes in the control and Robo4 vaccinated mouse sera. Sera, harvested at day 28 from Fc or Robo4-Fc vaccinated mice, was diluted tenfold and used in an ELISA with secondary antibody specific for mouse IgG1, 2a, 2b, 3 or IgM.

Figure 2

Robo4 vaccination inhibited fibrovascular invasion and angiogenesis in subcutaneously implanted sponges

Mice were vaccinated with either Fc or Robo4-Fc and sponges were implanted at day 28. 2 ng of basic fibroblast growth factor (bFGF) was injected into the sponges on alternate days and they were harvested after 14 days. **(a)** Representative images of sections of the implanted sponges stained with haematoxylin and eosin (H&E) (top panels). Using ImageJ image analysis software invaded areas were marked in red (bottom panels) **(b)** Quantification of the invaded area of the sponge. Invaded area as a percentage of the whole sponge was plotted for a representative section for each mouse. The mean and SEM are indicated ($P = 0.0006$, Mann-Whitney test). **(c)** Representative H&E stained sections show fewer and smaller vessels in Robo4-Fc vaccinated mice compared with control Fc vaccinated mice. **(d)** Vessel numbers from 3 random sections per mouse were quantitated and plotted, the mean and SEM are indicated ($p < 0.001$, Mann-Whitney test). **(e)** The area of 10 random vessels per group

was quantitated using ImageJ and plotted. The mean and SEM are shown ($p < 0.001$, Mann-Whitney test).

Figure 3

Robo4 vaccination retarded tumor growth and increased fibrinogen deposition and neutrophil infiltration

Mice were vaccinated with either Fc or Robo4-Fc and subcutaneously implanted with 10^6 Lewis lung carcinoma cells. The size of tumor was measured three times per week. **(a)** Tumor volume was plotted and Two-way ANOVA analysis of tumor volume was performed ($P < 0.05$, $n = 6$ per group). **(b)** Immunofluorescent staining of fibrinogen was performed using polyclonal fibrinogen antisera on 4 sections for 4 tumors per group. Quantification of the area positive for fibrinogen staining (green) was determined using ImageJ. The mean and SEM are indicated, ($p < 0.001$, Mann-Whitney test). **(c)** Neutrophil invasion was assessed by immunofluorescent staining with monoclonal anti-Ly6G and Ly-6C antibody. The number of infiltrating neutrophils (red) was counted from 4 sections from each of 4 tumors. The mean and SEM are indicated, ($p < 0.001$, Mann-Whitney test).

Figure 4

Robo4 vaccination with alum adjuvant retarded tumor growth

(a) Alum vaccination protocol: mice were vaccinated with either Fc or Robo4-Fc with alum as an adjuvant. 10^6 Lewis lung carcinoma cells per mouse were concurrently implanted subcutaneously. Tumor growth in Fc or Robo4-Fc alum vaccinated wild type C57/BL6 mice. Tumor volume was plotted as mean ±SEM. Two-way ANOVA analysis of tumor volume was performed ($P < 0.001$, $n = 7$ per group). **(b)** Robo4 antibodies were determined by ELISA at day 21 post-immunisation. The antibody titer was normalized to positive control serum from Freund's adjuvant vaccinated mice. The mean and standard error of the mean (SEM) are indicated, ($n = 7$ per group, $p < 0.01$, Mann-Whitney test). **(c)** CD8 depletion protocol: Mice were vaccinated with either Fc or Robo4-Fc with alum as an adjuvant. CD8 depletion was initiated 14 days later with a 3 day interval between injections of anti-CD8

antibodies. Tumor growth in control mice and in mice depleted of $CD8^+$ cells. Tumor volume was plotted as mean ±SEM. Two-way ANOVA analysis of tumor volume was performed (Control group: $P < 0.001$, n = 6 per group; $CD8^+$ depletion group: $P < 0.005$, n = 6 per group). **(d)** Robo4 antibodies were determined by ELISA at day 21 post-immunisation. The antibody titer was normalized to positive control serum from Freund's adjuvant vaccinated mice. The mean and standard error of the mean (SEM) are indicated, (n = 6 per group, $p < 0.01$, Mann-Whitney test). **(e)** Tumor growth in Fc or Robo4-Fc alum vaccinated B cell knockout mice. Tumor volume was plotted as mean ±SEM (n = 6 per group) **(f)** Tumor growth in Fc or Robo4-Fc alum vaccinated IgG1 knockout mice. Tumor volume was plotted as mean ±SEM (n = 6 per group).

Figure 5

Induction of protective Robo4-specific antibodies in carrier-primed animals

(a) Carrier priming strategy for rapid production of Robo4 antibodies. Pre-vaccination of a carrier protein in a strong adjuvant ensures the presence of carrier-specific memory T cells, which allows rapid Robo4 antibody production upon subsequent exposure to Robo4-carrier conjugates. **(b)** Mice receiving primary immunisation with human Fc in alum were reimmunised 5 weeks later with mouse Robo4-Fc protein without adjuvant. Concurrently 10^6 Lewis lung carcinoma cells were implanted subcutaneously. Tumor growth in Fc or Robo4-Fc alum vaccinated Fc primed mice. Tumor volume was plotted as mean ±SEM. Two-way ANOVA analysis of tumor volume was performed ($P < 0.001$, n = 10 per group). **(c)** Robo4 antibodies were determined by ELISA at day 21 post-immunisation. The antibody titer was normalized to positive control serum from Freund's adjuvant vaccinated mice. The mean and standard error of the mean (SEM) are indicated, (n = 10 per group, $p < 0.001$, Mann-Whitney test). **(d)** Mice receiving initial immunisation with chicken gamma globulin (CGG) in alum (29) were reimmunised 5 weeks later with Robo4-Fc cross-linked with CGG using glutaraldehyde (21). Concurrently 10^6 Lewis lung carcinoma cells were implanted subcutaneously. Tumor growth in Fc or Robo4-Fc alum vaccinated CGG primed mice. Tumor volume was plotted as mean ±SEM. Two-way ANOVA analysis of tumor volume was performed ($P < 0.001$, n = 8 per group). **(e)** Robo4 antibodies were determined by ELISA at day 21 post-immunisation. The antibody titer was normalized to positive control serum from

Freund's adjuvant vaccinated mice. The mean and standard error of the mean (SEM) are indicated, (n = 8 per group, $p < 0.001$, Mann-Whitney test).

Figure 6

No pathological changes in a range of organs were observed after vaccination with Robo4.

(a) Quantification of the wound area (n = 6 per group). **(b)** H&E staining on heart, lung, kidney, spleen, brain and liver sections from Fc and Robo4-Fc vaccinated mice 6 weeks post vaccination.

Supplemental figure 1

Sequence of mouse Robo4-Fc and the site of papain cleavage

The extracellular domain of mouse Robo4 (Black) was fused to a human Fc (Blue) and the recombinant protein produced for vaccination in mouse. The papain cleaved product containing Robo4 alone was used in the ELISA as the coating antigen. The site of papain cleavage is indicated in red.

Supplemental figure 2

Production of extracellular Robo4 protein and papain cleavage of Fc. Development of an ELISA to measure anti-Robo4 antibody

HEK293T cells were transduced with lentivirus to produce human or mouse Robo4 extracellular domain-Fc. Fusion proteins were secreted into and purified from the tissue culture medium. Conditioned medium was collected on alternate days and purified by affinity chromatography using a protein A column. **(a)** SDS-PAGE and Coomassie staining of mouse Robo4-Fc purified from media harvested on alternate days. **(b)** Purified Robo4-Fc was treated with papain to cleave the Fc portion, which was depleted using protein A chromatography. The intact and purified mouse Robo4 extracellular domain was subject to SDS-PAGE and western blotted for both anti-mouse Robo4 or anti-Fc, showing effective depletion of the Fc portion. **(c)** The purified mouse or human extracellular domain was immobilized on ELISA plates at the indicated concentrations and a range of concentrations of either anti-human Robo4 (MR7 (22)) or anti-mouse Robo4 were tested to determine the sensitivity of the ELISAs.

Supplemental figure 3

Expression of Robo4 on vessels in implanted sponges and Lewis lung carcinoma tumors

(a) Immunofluorescent staining was performed on the sponges of the control mice. Robo4 was stained using a mouse Robo4-specific antibody, the endothelial cells were stained with *Bandieria simplicifolia* isolectin B4-FITC and DAPI was used to visualize nuclei (blue). Fluorescence detected within the vessel lumen is due to autofluorescence of erythrocytes in

both red and green channels which is absent from the blue channel. Images were acquired using confocal microscopy. **(b)** Immunohistochemistry was performed to determine Robo4 expression in Lewis lung carcinoma tumors. The primary antibodies were anti-CD31 which stains all endothelial cells and anti-mouse Robo4. A similar staining pattern was observed in the tumor vasculature for both markers.

Supplemental figure 4

Comparative analysis of sera from Freund's and alum vaccinated mice.

Determination of Robo4-specific antisera in Freund's and alum vaccinated mouse sera. Mouse sera harvested at day 28 post-vaccination, was diluted tenfold and used in an ELISA. Commercial mouse Robo4-his protein (Sino Biological Inc. China) was used as the coating antigen in this assay.

Supplemental figure 5

***In vivo* depletion of $CD8^+$ cells in vaccinated mice**

Depletion of $CD8^+$ cells was initiated on the day of tumor challenge by injection of anti-CD8 antibodies. The depletion was verified by immunostaining of peripheral blood cells using anti-mouse CD3-PECy5 and anti-mouse CD8-FITC antibodies 10 and 20 days post-depletion.